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Purification and characterization of a specific late-larval esterase from two species of the *Drosophila repleta* group: contributions to understand its evolution

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Abstract

Background: After duplication, one copy of an original gene can become redundant and decay toward a pseudogene status or functionally diverge. Here, we performed the purification and biochemical characterization of EST-4 (a late larval β-esterase) from two *Drosophila repleta* group species, *Drosophila mulleri* and *Drosophila arizonae*, in order to establish comparative parameters between these enzymes in these species and to contribute to better understand their evolution.

Results: In *D. mulleri*, EST-4 had an optimal activity in temperatures ranging from 40° to 45°C and at pH 7.5, maintaining stability in alkaline pH (8.0 to 10.0). It was classified as serine esterase as its activity was inhibited by PMSF. No ion negatively modulated EST-4 activity, and iron had the most positive modulating effect. In *D. arizonae*, it showed similar optimum temperature (40°C), pH (8.0), and was also classified as a serine esterase, but the enzymatic stability was maintained in an acidic pH (5.5 to 6.5). Fe^{+2} had the opposite effect found in *D. mulleri*, that is, negative modulation. Al^{+3} almost totally inhibited the EST-4 activity, and Na^{+} and Cu^{+2} had a positive modulation effect. Kinetic studies, using ρ-nitrophenyl acetate as substrate, showed that EST-4 from *D. mulleri* had higher affinity, while in *D. arizonae*, it showed higher V_{max} and catalytic efficiency in optimal reaction conditions.

Conclusions: EST-4 from *D. mulleri* and *D. arizonae* are very closely related and still maintain several similar features; however, they show some degree of differentiation. Considering that EST-4 from *D. mulleri* has more conspicuous gel mobility difference among all EST-4 studied so far and a lower catalytic efficiency was observed here, we proposed that after duplication, this new copy of the original gene became redundant and started to decay toward a pseudogene status in this species, which probably is not occurring in *D. arizonae*.

Keywords: β-esterase; EST-4; *D. mulleri*; *D. arizonae*; Evolutionary biochemistry

Background

Several *Drosophila* species hold a class of β-esterase isoenzymes that have been reported to be encoded by a cluster of genes that are products of gene duplications (Zouros et al. 1982; Collet et al. 1990; East et al. 1990; Brady and Richmond 1992; Karotam et al. 1993; Oakeshott et al. 1999; Balakirev et al. 2005; Robin et al. 2009). These enzymes have been studied regarding their biochemistry and genetic and evolutionary aspects, including some differences in enzymatic and biochemical properties (Zouros and van Delden 1982; Pen et al. 1984, 1986a, 1990; Korochkin et al. 1990; Brady and Richmond 1990; Richmond et al. 1990; Mateus et al. 2009, 2011), amino acid (Pen et al. 1986b, Pen et al. 1990) and gene sequencing (Balakirev et al. 2003; Robin et al. 2009), and gene localization in the chromosome (Gomes and Hasson 2003). In general, with some exceptions, two enzymes are detected in most species that hold this gene cluster, one expressed during all insect's life cycle and present mostly

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in the hemolymph, and other expressed in the late larvae and early pupae in the integument.

The more intensively β-esterase genes studied are *Est6* and *Est7* of *Drosophila melanogaster* subgroup (Oakeshott et al. 1995, 1999; Balakirev et al. 2006). They are tightly linked, and *Est6* has acquired a novel function in this set of species (Oakeshott et al. 2000). In *D. melanogaster*, it is highly expressed in the sperm ejaculatory duct of the adult male instead of in the hemolymph, and the enzyme is transferred to the female during mating and modifies her subsequent egg-laying and remating behaviors (Meikle et al. 1990; Richmond et al. 1990; Saad et al. 1994). *Est7* is predominantly expressed in the integumental tissue of late larvae and early pupae (Dumancic et al. 1997), and its function there still remains unknown.

In other species of the genus, the β-esterase gene cluster seems to be more complex in composition and function. In *Drosophila pseudoobscura* (*obscura* group, also *D. melanogaster*'s subgenus *Sophophora*), three *in tandem* genes are found, and they show evidences of gene conversion or reciprocal recombination (Brady and Richmond 1992; King 1998). One of these three encodes the major adult hemolymph β-esterase (as *D. melanogaster*'s *Est6* above), but no function was detected for the other two genes (Brady and Richmond 1990; Tamarina et al. 1997). In *Drosophila virilis* (*virilis* group) and several cactophilic species (*repleta* group) of the subgenus *Drosophila*, the basic adult hemolymph and preadult integument β-esterases have been detected, and at least one and at most three β-esterases are present in the male ejaculatory bulb (Oakeshott et al. 1990, 1993). In *Drosophila mojavensis*, Robin et al. (2009) identified six paralog genes belonging to the β-esterase cluster and annotated, based on amino acid (Pen et al. 1986b) and gene sequence comparisons, that the isoenzymes, named EST-4 and EST-5 (Zouros et al. 1982), are probably the products of *Est2c* and *Est2a* genes, respectively. These enzymes have the same substrate preference for β-naphtyl esters (Zouros and van Delden 1982; Pen et al. 1984; Mateus et al. 2011), but as expected by default, they are highly differentiated about their temporal and tissue expression patterns. EST-4 is found only during the late larval stage and manly in the larvae cuticle, and EST-5 is present during throughout the insect's life cycle and predominantly in the hemolymph and fat body (Zouros et al. 1982; Pen et al. 1984). *Drosophila buzzatii*, a South American cactophilic species, also has very closely linked loci in chromosome 2 encoding esterase isozymes with the typical hemolymph and late larval/early pupal cuticle expression profiles (East et al. 1990; Gomes and Hasson 2003).

In a more specific analysis in the *repleta* group, Zouros et al. (1982) detected EST-5 activity in all the 13 species they analyzed, but apparently, this was not true for EST-4. The activity of β-esterase in the larval carcass varied considerably among the species. It was abundant in most species, but it was barely detected in three (*Drosophila aldrichi*, *D. repleta*, and *Drosophila peninsularis*) and totally missing in other three (*Drosophila tira*, *Drosophila hydei*, and *Drosophila eohydei*). Mateus et al. (2011) studied six species of the same group of species and detected EST-4 and EST-5 activity in all the species: *D. mojavensis* cluster species (*D. mojavensis*, *D. arizonae*, and *Drosophila navojoa*) showed fainter bands than *D. mulleri* cluster species (*D. mulleri*, *D. aldrichi*, and *Drosophila wheeleri*).

A number of other results have shown that EST-4 and EST-5 are closely related, but with differences other than those already presented above. They have similar isoelectric points (between 6.0 and 7.0; Mateus et al. 2011), exhibit 82% identity in N-terminals of amino acid sequences (Pen et al. 1986b), and form an interloci heterodimer (Zouros et al. 1982; Mateus et al. 2011). However, they have different molar masses (Pen et al. 1984, 1986b; Mateus et al. 2009, 2011) and exhibit differential inhibition profiles, with EST-4 being inhibited by phenylmethanesulfonyl fluoride (PMSF) and not affected by malathion, and EST-5 being inhibited by malathion and not affected by PMSF (Mateus et al. 2011).

After duplication, a new copy of an original gene can take two possible pathways. It can become redundant and decay toward a pseudogene status or it functionally diverges. Gene duplication followed by functional divergence has been long considered the primary mechanism of molecular evolution (Lewis 1951; Ohno 1970). Balakirev and Ayala (1996) have detected high frequencies of null alleles (more than 60%) for *Est7* gene, which encodes the integumental tissue of late larvae and early pupae enzyme in *D. melanogaster*. The two explanations above have been suggested for this result. Balakirev and Ayala (2003) and Balakirev et al. (2006) proposed that the EST-7 protein has become redundant, and the gene is decaying toward a pseudogene status. Alternatively, Balakirev and Ayala (2004) suggested that the *Est7* gene maintains a function that is not disabled by the stop codons or frame-shifting mutations detected.

According to Lima-Catelani et al. (2004), differences in esterase synthesis during the insect life cycle are probably due to differences in the regulatory mechanisms acting accordingly with metabolic function requirements of a variable number of processes in which esterases are involved during development. On the other hand, Robin et al. (2009) detected contrasting examples of the types and stages of loss of gene function for β-esterases as they inferred missing orthologs, pseudogenes, and null alleles, and a minimum of nine gene gain-loss events in the 12 species genome analyzed. They speculated that

their results are probably related to fluctuation in the requirements for the functions of these genes over evolutionary time, possibly in response to changes in environmental niches. However, the reproductive functions of some β-esterases suggest that the copy number could have been changed by sexual competition or conflict.

Thus, this work aimed to investigate differences and compare several biochemical and enzymatic properties of EST-4 in two *Drosophila* species from the *repleta* group, *D. mulleri* and *D. arizonae*, in order to contribute to better understand the differentiation and evolution of this enzyme. Preliminary characterization of this enzyme showed, in *D. mulleri*, that it has the most different electrophoretic pattern from all other species analyzed so far, aside from some other distinct enzymatic features such as having the highest isoelectric point and molecular weight (Mateus et al. 2011). According to Harms and Thornton (2013), an integration of evolution with biochemistry is indispensable to achieve a more complete understanding of why biological molecules have the properties that they do. Our results are in agreement to previous achievements, and the lower V_{max} and catalytic efficiency detected for *D. mulleri* lead us to propose that after duplication, this copy of the original gene became redundant and started to decay toward a pseudogene status in this species, which probably is not occurring in *D. arizonae*.

Methods

Species

Multifemale lineages of the two species, *D. mulleri* and *D. arizonae*, were obtained from Prof. Dr. Carlos Roberto Ceron (Department of Chemistry and Environmental Sciences, IBILCE/UNESP, São José do Rio Preto, Brazil). They were maintained as mass cultures in 250-mL culture vials with standard banana agar medium in constant temperature of 25°C ± 1°C and 12-h photoperiod.

EST-4 purification

Sample preparation

Late-third instar larvae of both species were obtained directly from the maintenance vials, and in order to maximize sample attainment, intraspecific crosses were performed. Virgin males and females were separated, and after 7 days, ten vials containing standard culture medium were prepared. Five couples were crossed for 21 days, transferring them into new vials every 7 days. All vials were daily checked for tracking larval development, separating those larvae that were at the desired stage.

The larvae collected were immediately frozen in liquid nitrogen and stored at −80°C. The enzyme extracts were obtained by macerating 400 late-third instar larvae in 0.1 M phosphate buffer at pH 6.2 and centrifuging at 10,000 × *g* in 4°C for 10 min. A sample of each supernatant was electrophoresed in 10% non-denaturing polyacrylamide gel, as described by Mateus et al. (2011) and adapted in 'Molar mass exclusion and ion exchange chromatographies' section below, in order to detect the presence of EST-4. An adult was used as a comparative sample.

Molar mass exclusion and ion exchange chromatographies

The purification in gel filtration through molecular mass exclusion chromatography (MMEC) was performed using Sephadex G-75 resin (GE Healthcare, São Paulo, Brazil), which was packed into a 4 × 100-cm (diameter × height) column. Fractionation was performed at 4°C using 0.1 M phosphate buffer at pH 6.2 and a 0.6-mL/min flow. Fractions of 5 mL were collected and individually analyzed for larval esterase activity in polyacrylamide gel electrophoresis (PAGE), mixing 20 μL of the fraction with 5 μL sample buffer (25% Tris–HCl buffer (0.05 M) pH 6.8, 20% glycerol, and 0.02% bromophenol blue). The electrophoresis was performed in 10% PAGE as described by Mateus et al. (2011) at constant voltage of 110 V in room temperature. To test the substrate specificity, the gels were soaked with usual α- and β-naphthyl acetate solution (Mateus et al. 2011), and their products were stained for 2 h using Fast Blue RR salt (Sigma-Aldrich, São Paulo, Brazil).

The fractions that showed EST-4 activity were joined and dialyzed in 20 mM *N*-[tris(hydroxymethyl)methyl]-3-aminopropanesulfonic acid (TAPS) buffer at pH 8.5 for 24 h at 4°C and with three exchanges. The dialyzed material was submitted to ion exchange chromatography (IEC) in a Q-Sepharose resin (GE Healthcare, São Paulo, Brazil) with the same buffer above. The sample was washed with elution buffer (20 mM TAPS buffer of pH 8.5 without NaCl) to remove unbound material. The elution of proteins was initiated with linear salt gradient ranging from 0 to 2 M NaCl in the same buffer. Fractionation was performed at 4°C with a 1.0-mL/min flow, and 5-mL fractions were collected.

After each chromatography, EST-4 purity was certified through denaturing gel electrophoresis (10% sodium dodecyl sulfate (SDS)-PAGE) according to Laemmli (1970). The samples (20 μL) were mixed with 10 μL sample buffer (25% Tris–HCl buffer (0.05 M), pH 6.8, 3.1% DTT (*w*/*v*), 0.02% Bromophenol Blue, 20% glycerol, and 4% SDS (*w*/*v*)). This mixture was boiled for 5 min at 96°C, and after electrophoresis, the gel was stained with silver nitrate (See and Jackowski 1989).

EST-4 activity test

The esterase activity was measured using the protocol described by Immanuel et al. (2010) with some modifications: 25 μL of enzyme extract, 25 μL of 0.05 M 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) buffers

(pH 7.0 for *D. mulleri* and pH 7.5 for *D. arizonae*), and 450 μL of ρ-nitrophenyl as substrate. The unit of the esterase activity used was the equivalent to one micromole of ρ-nitrophenyl released per unit of time (minute) under standard test conditions, that is, the amount of enzyme required to cause an increase of $0.001A_{280nm}$. All assays were performed in triplicate. The calculation of esterasic unit was realized according to Semionato (2006), in which the unit of enzyme is given by the following formula:

$$U = \frac{\text{Abs} \times V_R \times 1,000}{e \times t \times V_E},$$

where, Abs means absorbance at 410 nm, V_R is the volume of reaction which is 500 μL, e is the molar extinction coefficient of ρ-nitrophenyl which is 18.5 μmol mL^{-1} cm^{-1}, t is time in minutes, and V_E is the volume of enzyme which is 25 μL.

Protein quantification

The esterase quantification was determined according to the method described by Bradford (1976), using standard curve constructed with bovine serum albumin (BSA).

Biochemical characterization of EST-4

pH effect on the EST-4 activity and stability

Optimum pH and pH stability characterization of both enzymes were carried out with pH ranging from 4.5 to 10.5, varying 0.5 U. The following buffers were used: acetate (pH 4.5 and 5.0), 2-[*N*-morpholino]ethanesulfonic acid (MES; pH 5.5, 6.0, and 6.5), HEPES (pH 7.0, 7.5, and 8.0); *N*,*N*-bis(2-hydroxyethyl)glycine (BICINE; pH 8.5 and 9.0), and 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS; pH 9.5, 10.0, and 10.5). All the buffers were prepared with 0.05 M of concentration. The reaction mixes contained 25 μL of purified enzyme, 25 μL of buffer (ranging from pH 4.5 up to 10.5, described above), and 450 μL of ρ-nitrophenyl acetate substrate. Thus, thirteen different mixes were set up, each one with different final pH, and they were incubated at 40°C for 30 min. After this period, the esterase activity was measured accordingly to the method described above ('EST-4 activity test' section).

The pH stability was determined by incubating the enzymes (25 μL) for 1 h at 25°C at different pH values (using the buffers described above), subsequently adding 450 μL of ρ-nitrophenyl acetate substrate, 13 μL of optimum pH buffer (pH 6.5 to *D. mulleri* and pH 7.5 to *D. arizonae*), and determining the activity as described above at 40°C for 30 min.

Temperature effect on the EST-4 activity

The influence of temperature on the activity of EST-4 was performed in optimal pH (7.0 for *D. mulleri* and 7.5 for *D. arizonae*) and temperature of 25°C to 55°C, with variations of 5°C. The pure enzymes (25 μL) were mixed with 25 μL of optimum pH buffer and 450 μL of ρ-nitrophenyl acetate substrate, as described in the 'EST-4 activity test' section. The enzyme activities were evaluated proceeding incubation for 30 min in the respective temperatures.

Chemical effect on the EST-4 activity

The determination of active site constitution of both enzymes was performed according to the protocol described by Dunn (1989) with modifications. The following reagents were used in a final concentration of 5 mM: PMSF, EDTA (ethylenediamine tetraacetic acid), and pepstatin.

The pure enzymes (25 μL) were premixed with 2.5 μL of each inhibitor, incubated at 40°C for 5 min, and after that period, 22.5 μL of optimum pH buffer and 450 μL of ρ-nitrophenyl acetate substrate were added. The enzyme activities were checked by incubating them for 30 min at 40°C. The control tube was made with addition of 25 μL pure enzyme, 450 μL of ρ-nitrophenyl acetate, and 25 μL of optimum pH buffer.

The effects of metal ions on the esterase activities were investigated by adding monovalent (Li^+, Na^+, and K^+) and divalent ions (Ba^{2+}, Ca^{2+}, Mg^{2+}, Mn^{2+}, Fe^{2+}, Ni^{2+}, Cu^{2+}, and Zn^{2+}) to a final 10 mM concentration. The pure enzymes (25 μL) were preincubated with each ion (2.5 μL) at 40°C for 5 min. Subsequently, the enzyme activities were evaluated at 40°C for 30 min and added with 450 μL of ρ-nitrophenyl acetate substrate and 22.5 μL of optimum pH buffer. The control tube was made with the addition of 25 μL pure enzyme, 450 μL of ρ-nitrophenyl acetate, and 25 μL of optimum pH buffer.

EST-4 enzymatic kinetics

The enzyme kinetics was obtained for both EST-4 by adding increasing concentrations of ρ-nitrophenyl acetate substrate, from 0.1 to 1.0 mM. The experiments were performed in the optimum pH and at 40°C, and the results were read in a spectrophotometer with 410-nm absorbance.

The K_m and V_{max} kinetic values were obtained using Michaelis-Menten equation calculated by non-linear regression of data from hydrolysis of the substrate using the software GraFit version 5.0 (Erithacus Software Ltd., Surrey, UK). K_m, K_{cat}, and K_{cat}/K_m were evaluated by determining the enzyme activities against ρ-nitrophenyl acetate substrate in ideal conditions.

Results and discussion

Purification of EST-4 from *D. mulleri* and *D. arizonae*

Chromatography

After gel filtration through MMEC, two protein peaks with esterase activity were detected in *D. mulleri*

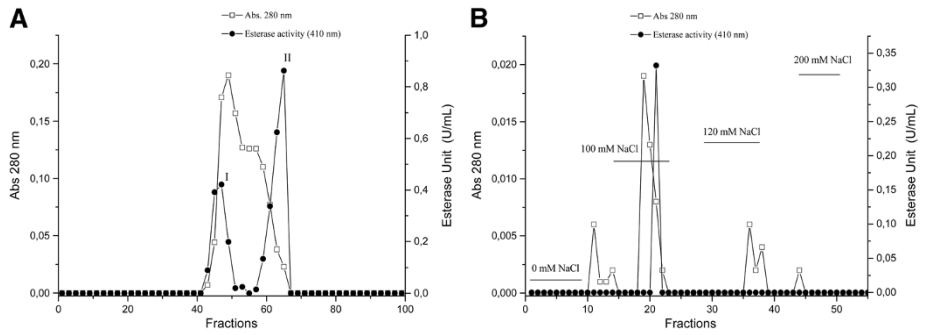

Figure 1 Elution profiles for *Drosophila mulleri*. MMEC-Sephadex G-75 **(A)** and IEC-Sepharose Q **(B)**.

(Figure 1A) and *D. arizonae* (Figure 2A). The elution profile of the fractions was analyzed in 10% PAGE, using a larva as control (Figure 3). According to Zouros et al. (1982), Pen et al. (1984), and Mateus et al. (2011), EST-4 shows preference for β-naftil acetate, therefore stains in pink, which was observed in the gels. EST-4 was found in peak II in *D. mulleri* and peak I in *D. arizonae*. Fractions that showed EST-4 activity in the gel were pooled and submitted to SDS-PAGE to certify the degree of enzyme purification. The results showed partial purification of EST-4 (results not shown). The pooled fractions were dialyzed, concentrated, and submitted to IEC to enhance enzyme purity. After this second chromatography, the elution profile of the fractions was again analyzed in 10% PAGE using a larva as control (results not shown). EST-4 from *D. mulleri* was detected in the protein peak of 100 mM of NaCl (Figure 1B) and from *D. arizonae* in the protein peak of 200 mM of salt (Figure 2B). The certification of EST-4 purity in the fractions was confirmed by SDS-PAGE (Figure 4). Those containing purified EST-4 were pooled and concentrated for its further biochemical characterization.

The molar mass of EST-4 was determined using protein molecular weight marker (low molecular weight SDS calibration kit for SDS electrophoresis, GE Healthcare) in the SDS-PAGE gel. As seen in Figure 4, the molar masses of the purified EST-4 (correspondent to the purified protein subunit (arrow)) of *D. mulleri* and *D. arizonae* are approximately 45 kDa. Pen et al. (1984) determined the molar mass of EST-4 of *D. mojavensis* also using denaturing gel electrophoresis (SDS-PAGE) and obtained values between 62 and 64 kDa for its subunits. Similarly, the molar mass of 64 to 66 kDa for the subunits of EST-5 of the same species was found by Pen et al. (1986a). As pointed out before, EST-4 and EST-5 are expressed by duplicated genes, *Est2c* and *Est2a*, respectively, in these species. Mateus et al. (2011) and Pen et al. (1984) have determined the molar mass of the dimeric EST-4 protein as being between 83 and 95 kDa. Therefore, our results showed a more congruent data for the molar mass of the subunit, as it appears to have half the mass of the dimeric protein, and not the anomalous behavior previously found by Pen et al. (1984, 1986a).

The experimental purification data are summarized in Table 1. After the first step of the purification procedure, MMEC, the esterases from *D. mulleri* were purified 2.41-fold, and 5.17% recovery was obtained. After the second step of purification, IEC, EST-4 was purified 3.37-fold with a total of 3.61% recovery. *D. arizonae* showed better results, with a 1.67-fold esterase purification and 52.2% recovery after MMEC, and 3.14-fold

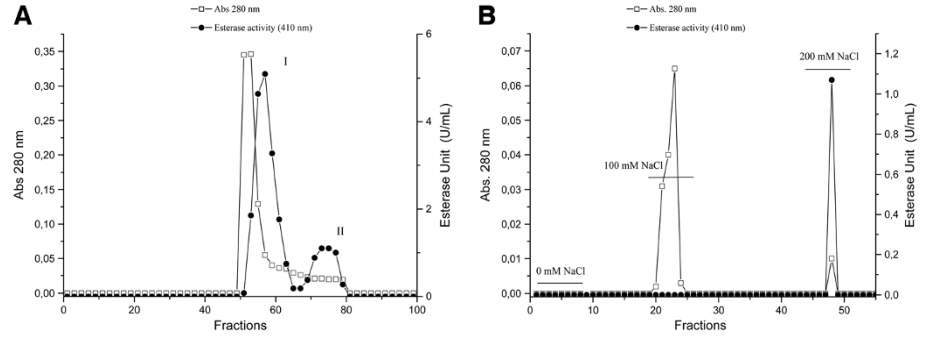

Figure 2 Elution profile for *Drosophila arizonae*. MMEC-Sephadex G-75 **(A)** and IEC-Sepharose Q **(B)**.

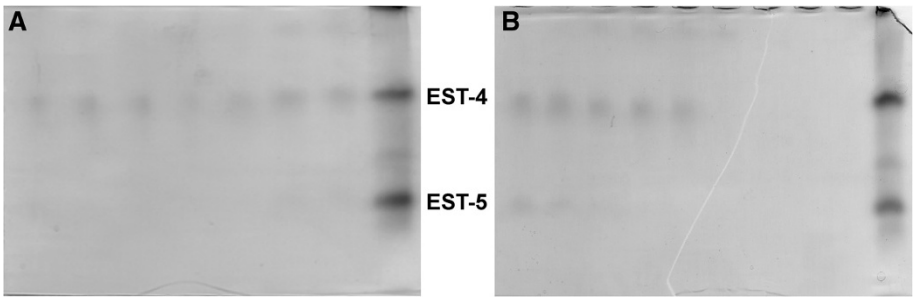

Figure 3 Esterase activity in 10% PAGE after MMEC. (A) *Drosophila mulleri*. **(B)** *Drosophila arizonae*.

purification and 6.65% recovery after IEC. Therefore, EST-4 recovery after the purification process in *D. arizonae* was approximately twice higher than that in *D. mulleri*. Similar results were obtained in a study involving purification and characterization of EST-5 from *D. pseudoobscura*, also classified as β-esterase, by Narise and Hubby (1966) who obtained 3.0-fold purification and 2.4% recovery of this enzyme. However, other works obtained much higher recovery after esterase purification. Pen et al. (1984) obtained 820-fold purification and 27% recovery of EST-4 activity from *D. mojavensis*. In other insects, Zera et al. (2002) purified and characterized the juvenile hormone esterase (JHE) from *Gryllus assimilis*, and after four steps of purification, they obtained more than 900-fold purification and 30% enzyme activity recovery. These much higher purification results are probably related to the amount of initial sample obtained for protein extraction (1 g of larvae in the case of Pen et al. 1984) and/or differences in the purification procedures applied (seven steps in Pen et al. 1984, while four steps in Zera et al. 2002).

Biochemical characterization of EST-4

Effect of pH on the EST-4 activity and stability

EST-4 of both species had similar activity profiles on different pH buffers. The optimum activities were detected in alkaline pH, 7.5 for *D. mulleri* (Figure 5A) and 8.0 for *D. arizonae* (Figure 5B). These results showed that these enzymes have the best structural conformation state, and consequently better catalytic performance, almost at the same pH. However, the enzyme activity profiles in the surrounding pH had differences. EST-4 of *D. mulleri* showed around 40% to 60% of its optimum activity in the pH levels of 7.0, 8.0, 8.5, and 9.0; result not observed for EST-4 from *D. arizonae*, which displayed 40% to 60% of its activity only in the nearest optimum pH, 7.5 and 8.5.

The enzyme stability tests also showed that EST-4 from both species had differences. EST-4 of *D. mulleri* had lower residual esterase activity in an acid pH, maintaining approximately 80% of its activity in pH between 5.0 and 6.5 (Figure 6A). However, the higher the pH, the higher the enzyme stability, and this enzyme had more stable activity in alkaline pH levels, especially between 8.0 and 10.0. For *D. arizonae*, EST-4 showed lower stability in alkaline pH, retaining approximately 40% of its residual activity in the pH around 8.0 and 10.0, and it was more stable in acidic pH, especially around pH 5.5 and 7.0, where its activity was approximately 80% (Figure 6B).

Therefore, regarding pH, both EST-4 had similar optimum pH curve but different pH stability. Thomazine (2007) studied two enzymatic variants of EST-5, called fast (EST-5 F) and slow (EST-5S), and found that these allozymes presented pH profiles similar to that described here, i.e., optimum activity in alkaline pH and lower activity in acid pH. In a study of characterization of JHE in *D. melanogaster*, Campbell et al. (1992) found that below pH 6.0, considerable non-enzymic, acid hydrolysis of juvenile hormone (JH) occurred. Over the pH range from 6.0 to 8.6, the JHE activity almost doubled, increasing linearly with increasing pH. No further change in activity was observed at pH 9.0. Therefore, they showed that JHE from *D. melanogaster* also had a tendency of better activity on alkaline environment.

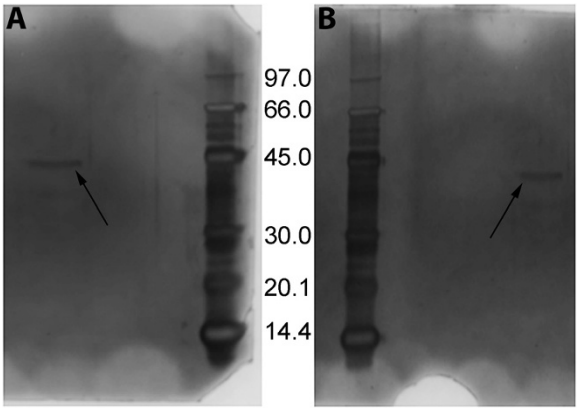

Figure 4 10% SDS-PAGE of purified EST-4 (arrows) from *D. mulleri* (A) and *D. arizonae* (B). Molecular weights between gels in kDa (1×10^{-3} Da).

Table 1 EST-4 purification features from *Drosophila mulleri* and *Drosophila arizonae* after MMEC and IEC

Purification steps	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Recovery (%)	Purification (fold)
D. mulleri					
Crude extract	0.1400	46.00	328.57	100.00	1.00
MMEC	0.0030	2.38	793.33	5.17	2.41
IEC	0.0015	1.66	1,106.66	3.61	3.37
D. arizonae					
Crude extract	0.1700	241.35	1,419.70	100.00	1.00
MMEC	0.0530	126.00	2,377.36	52.20	1.67
IEC	0.0036	16.05	4,458.33	6.65	3.14

Effect of temperature on the EST-4 activity

EST-4 from *D. mulleri* showed optimum temperatures of 40°C and 45°C (Figure 7A). Its activity decreased to about half when incubated at temperatures 5°C below (35°C) and above (50°C) these optimum temperatures. Temperatures below 35°C and above 50°C completely eliminated the enzyme activity. For *D. arizonae*, the EST-4 optimum temperature was 40°C (Figure 7B), decreasing to less than 80% of its activity when treated in 35°C, and to around 50% to 60% when treated in 45°C and 50°C. Temperatures below 35°C and above 50°C strongly affected the enzyme, remaining less than 20% of its original activity.

These results provide evidence that both enzymes had similar optimum temperatures, with minor differences as EST-4 from *D. mulleri* operated better at higher temperatures (45°C) when compared to *D. arizonae*. Thomazine (2007) found even higher optimum temperature in experiments with variants of EST-5 (slow and fast) of *D. mulleri*, which showed higher activity at 50°C. However, when these variants were incubated for 10 min at 55°C, no activity was detected for EST-5 F.

Effect of chemicals on the EST-4 activity

To classify the chemical nature of the catalytic site, i.e., the main residue composition in enzyme active site, the effect of the reagents was studied by incubating EST-4 in their presence. The essays showed that the largest reduction in esterase activity was caused by PMSF, approximately 85% for *D. mulleri* (Figure 8A) and 90% for *D. arizonae* (Figure 8B), suggesting that both belong to the class of serine esterases. According to Zhou et al. (1994), serine esterases catalyze the hydrolysis of esters and amides via covalent ester bound, which is formed between the substrate acyl portion and the serine residue of the enzyme active site.

These inhibition results corroborate the previously data of Mateus et al. (2011) who detected that the EST-4 of six species of the *D. repleta* group, including the two studied here, were all inhibited by PMSF. According to Dunn (1989), this type of inhibition probably occurs because this compound irreversibly binds to the hydroxyl side chain of the serine residue, impeding the enzymatic catalysis. This class of esterase is commonly found in insects (see Krejci et al. 1991, Anthony et al. 1995, Hinton and Hammock 2003, Coutinho-Abreu et al. 2007, Yu et al. 2009, and Li et al. 2010 as examples). However, in some insects, they were not detected. For example, in a study of biochemical identification and characterization of esterases in *Tribolium castaneum*, Gigliolli et al. (2011) observed that all enzymes were inhibited by eserine sulfate and/or malathion, and none by PMSF.

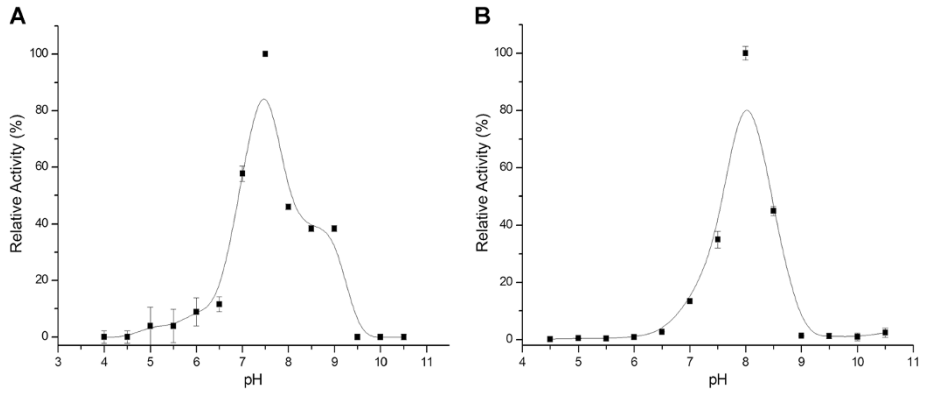

Figure 5 EST-4 activity in different pH levels. (A) *D. mulleri*. **(B)** *D. arizonae*.

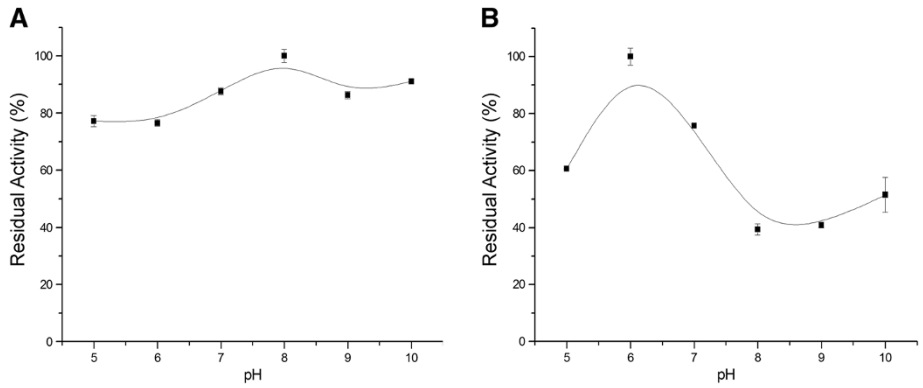

Figure 6 EST-4 stability in different pH levels. (A) *D. mulleri*. **(B)** *D. arizonae*.

Other two compounds also affected the EST-4 activity in our experiments. EDTA was responsible for more than 70% decrease in EST-4 activity in *D. arizonae* and about 35% decrease in the *D. mulleri*, suggesting that these esterases probably have their activity modulated positively in the presence of metal ions. In the presence of this compound, the ions were possibly chelated, resulting in the observed inhibition. Pepstatin was responsible for reducing approximately 20% of the enzymatic activity of EST-4 in both species. Serine enzymes commonly present a catalytic triad composed of serine, histidine, and aspartate residues, which indirectly interact, enhancing enzyme activity (Zhou et al. 1994). This inhibitor probably is connected to the aspartate residue causing the observed reduction in the EST-4 activity.

Considering the result with EDTA, the effect of metal ions on the activity of EST-4 of both species was also tested. These chemicals can be determinants in the protein molecular organization and can influence its enzymatic activity and stability. In *D. mulleri* (Figure 9A), Fe^{+2} increased fivefold the EST-4 activity compared to the control. Knowing that ions can bind to amino acids and influence protein structural conformation, directly affecting the catalytic performance of the enzyme (Merheb-Dini et al. 2009), we were able to propose that Fe^{+2} bound to EST-4 of *D. mulleri* and improved the enzyme activity probably because it made the enzyme structure better organized. Na^{+} and Ba^{+2} also showed a positive modulation effect, increasing around 50% of the EST-4 activity. These ions also probably linked to the enzyme, facilitating its catalytic function. Co^{+2}, Cu^{+2}, Li^{+}, and Ca^{+2} did not affect the enzymatic activity of EST-4 in this species. All other ions (Mg^{+2}, K^{+}, Mn^{+2}, Zn^{+2}, and Al^{+3}) promoted a small activating effect. It is noteworthy that none of the metal ions had negative modulating effect on this esterase activity.

In the case of *D. arizonae*, Na^{+} and Ba^{+2}, together with Cu^{+2} and Co^{+2}, also activated more than 50% (around 60%) of the EST-4 activity (Figure 9B). However, different from *D. mulleri*, several ions decreased the esterase activity, such as Fe^{+2}, Li^{+}, Mg^{+2}, Ca^{+2}, K^{+}, Mn^{+2}, Zn^{+2}, and Al^{+3}. This last one inhibited nearly 100% of the enzyme activity, and in this case, it is possible that it bound to the enzyme, promoting negative modulatory effect and preventing it to perform its catalytic function. Negative modulation of serine protease activity in the

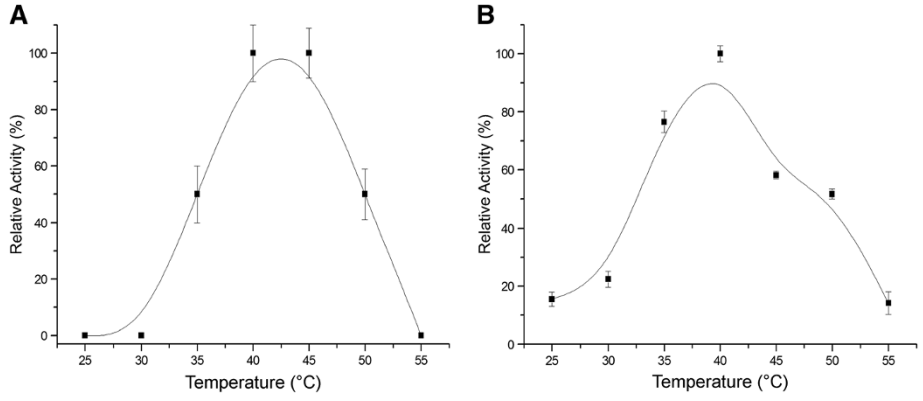

Figure 7 EST-4 activity in different temperatures. (A) *D. mulleri*. **(B)** *D. arizonae*.

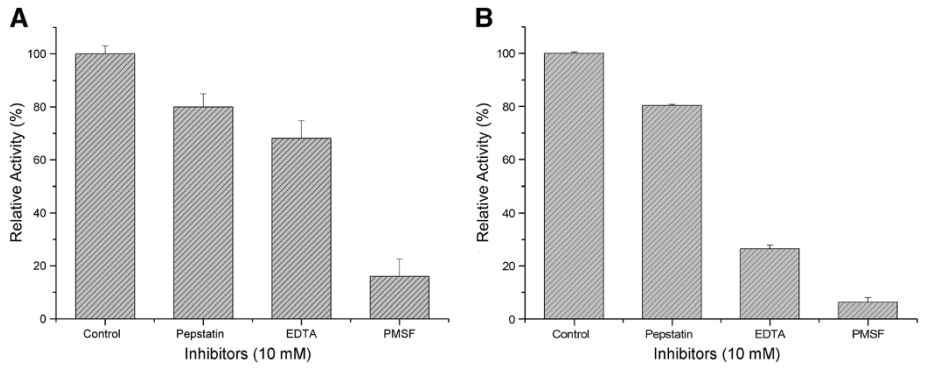

Figure 8 EST-4 activity in the presence of inhibitors. (A) *D. mulleri*. **(B)** *D. arizonae*.

presence of Al^{+3} was also observed by Silva (2011) in the fungus *Aspergillus fumigatus* Fresenius. They detected, among all ions tested, that the enzyme had its activity reduced about 80% only in the presence of this ion.

Therefore, the differences in the modulator effect of Fe^{+2} in both species are noteworthy. It was responsible for the activation of EST-4 in *D. mulleri*, and it had a negative modulatory effect in *D. arizonae*, decreasing approximately 40% of the esterase activity. Moreover, Al^{+3} showed an effect (positive or negative) over EST-4 of *D. mulleri* and was responsible for almost the complete inhibition of EST-4 in *D arizonae*. Thus, it can be suggested that although paralog genes with similar temporal and tissue expressions encode these enzymes, they are biochemically distinct in their catalysis regarding the presence of ions.

Kinetic parameters of EST-4

Kinetic parameters are peculiar and intrinsic in each enzyme/substrate relationship, depending entirely on the enzyme specificity. Studies about kinetic parameters of esterases, especially in *Drosophila* and using ρ-nitrophenyl acetate, are scarce and poorly described in the literature. In a study of purification and characterization developed by Zera et al. (2002), the JHE present in *Gryllus assimilis* hydrolyzed α-naphthyl and ρ-nitrophenyl esters, and according to these authors, the JHE studied probably can hydrolyze any ester with long aliphatic chains. Campbell et al. (1998) purified and characterized the *D. melanogaster* JHE, and it only hydrolyzed the substrate α-naphthyl acetate. In our case, both EST-4 depict the preference for β-naphthyl and were able to hydrolyze both α-naphthyl and ρ-nitrophenyl esters, as the JHE of *G. assimilis*. Using ρ-nitrophenyl as substrate, our results (Table 2) showed that EST-4 of *D. mulleri* had higher affinity for the substrate than *D. arizonae*. The K_m of EST-4 was much lower in the first species (0.17 mM) than in the second (0.74 mM), revealing that the concentration of ρ-nitrophenyl acetate needed for the enzyme to reach half of its maximum speed in *D. mulleri* is more than four times lower than that in *D. arizonae.* However, although the EST-4 affinity for this substrate in *D. mulleri* was much higher, its maximum velocity was approximately 20 times lower than in *D. arizonae* (V_{max}: *D. mulleri* = 0.94 mM min^{-1}, *D. arizonae* = 19.33 mM min^{-1}), having higher catalytic efficiency over the ρ-nitrophenyl acetate substrate.

Regarding the number of reaction cycles performed per unit of time, k_{cat}, it was possible to infer that the

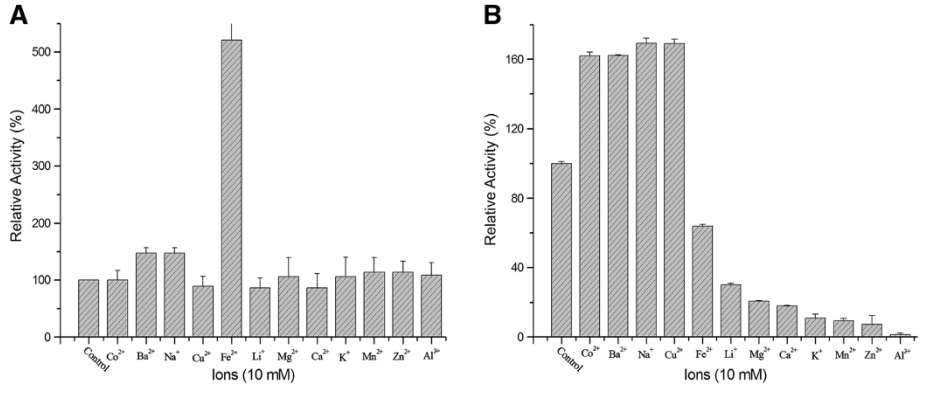

Figure 9 EST-4 activity in the presence of ions. (A) *D. mulleri*. **(B)** *D. arizonae*.

Table 2 EST-4 kinetic parameters using ρ-nitrophenyl acetate as substrate

Species	K_m (mM)	V_{max} (mM min^{-1})	k_{cat} (min^{-1})	k_{cat}/K_m (mM^{-1} min^{-1})
D. mulleri	0.17	0.94	94.54	532.0
D. arizonae	0.74	19.33	1,933.00	2,583.8

EST-4 of *D. arizonae* can convert the substrate into a product with higher efficiency (1,933 min^{-1}) as compared to the EST-4 of *D. mulleri* (94.54 min^{-1}). The catalytic efficiency (k_{cat}/K_m) corroborated these results as the EST-4 of *D. arizonae* showed higher catalytic efficiency (2,583.8 mM^{-1} min^{-1}) when compared to *D. mulleri* (532 mM^{-1} min^{-1}).

Conclusions

Our results clearly showed that these enzymes are very closely related and still maintain some similar features, such as optimal temperature and pH. However, they already depict many other characteristics that show they have differentiated in the evolutionary time (effect of chemicals, pH stability, enzymatic affinity, V_{max}, and catalytic efficiency). It seems that the EST-4 of *D. arizonae* is much better adjusted as an esterase enzyme than the EST-4 of *D. mulleri* because of its superior K_{cat} and K_{cat}/K_m. Considering that this enzyme of *D. mulleri* has more conspicuous difference in gel mobility among all EST-4 studied so far (Mateus et al. 2011) and its kinetic features observed here, it can be can propose that after duplication, one new copy of the original gene (in our case, the *Est2c* gene of EST-4) became redundant and started to decay toward a pseudogene status in this species, which probably is not occurring in *D. arizonae*. Balakirev and Ayala (1996) detected high frequencies of null alleles for the *Est7* gene, which encodes the enzyme found in the integumental tissue of late larvae and early pupae in *D. melanogaster*. This seems like to be the possible explanation for the observations detected for the EST-4 of *D. mulleri*.

Abbreviations

Abs: absorbance; BICINE: *N*,*N*-bis(2-hydroxyethyl)glycine; BSA: bovine serum albumin; CAPS: 3-(cyclohexylamino)-1-propanesulfonic acid; *e*: molar extinction coefficient of ρ-nitrophenyl; EDTA: ethylenediamine tetraacetic acid; HEPES: 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid; IEC: ion exchange chromatography; JHE: juvenile hormone esterase; kDa: kilodalton; MES: 2-[*N*-morpholino]ethanesulfonic acid; MMEC: molecular mass exclusion chromatography; NaCl: sodium chloride; PAGE: polyacrylamide gel electrophoresis; PMSF: phenylmethanesulfonyl fluoride; SDS: Sodium dodecyl sulfate; *t*: time in minutes; TAPS: *N*-[tris(hydroxymethyl)methyl]-3-aminopropanesulfonic acid; V_E: volume of enzyme; V_R: volume of reaction.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

VFL carried out all the biochemical analyses and drafted the manuscript. HC participated in the design of the study and helped in the biochemical analyses and in drafting the manuscript. LPBM participated in the design of the study and helped in drafting the manuscript. RPM conceived of the study, and participated in its design and coordination, and helped in drafting the manuscript. All authors read and approved the final manuscript.

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